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AUTHOR(S):

Soda, Kenji; Esaki, Nobuyoshi; Kurihara, Tatsuo; Liu, Ji-Quan

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Identification of Asp10 as the Active Site Nucleophile of L-2-Haloacid Dehalogenase of *Pseudomonas* sp. YL

Kenji Soda, Nobuyoshi Esaki, Tatsuo Kurihara and Ji-Quan Liu

L-2-Haloacid dehalogenase (EC class: 3.8.1.2) catalyzes the hydrolytic dehalogenation of L-2-haloalkanoic acids to produce the corresponding D-2-hydroxyalkanoic acids. We have analyzed the reaction mechanism of the enzyme from *Pseudomonas* sp. YL by means of ^{18}O incorporation experiment and tandem mass spectrometrical analysis of the labeled enzyme, and found that Asp10 is the active site nucleophile. Asp10 probably attacks the α-carbon of the substrate leading to the formation of an ester intermediate, which is hydrolyzed by nucleophilic attack of a water molecule on the carbonyl carbon atom.

Keywords: Dehalogenation / Haloalkanoic acid / Ionspray mass spectrometry

L-2-Haloacid dehalogenase (L-DEX) catalyzes the hydrolytic dehalogenation of L-2-haloalkanoic acids with inversion of the C_2-configuration producing the corresponding D-2-hydroxyalkanoic acids. We have isolated and purified thermostable L-2-haloacid dehalogenase (L-DEX YL) from a 2-chloroacrylate-utilizable bacterium, *Pseudomonas* sp. YL (1), cloned its gene (2), and constructed the overexpression system (3). The enzyme is composed of 232 amino acid residues (2), and its amino acid sequence is highly similar to those of L-DEXs from other bacterial strains and haloacetate dehalogenase H-2 from *Moraxella* sp. strain B (2).

Two different mechanisms have been proposed for the reactions of L-DEXs (Fig. 1) (4). According to the mechanism shown in Fig.1A, a carboxylate group of Asp or Glu acts as a nucleophile to attack the α-carbon of L-2-haloalkanoic acid, leading to the formation of an ester intermediate. This is hydrolyzed by an attack of water molecule activated by a basic amino acid residue of the enzyme. Alternatively, water is activated by a catalytic base of the enzyme, and directly attacks the α-carbon of L-2-haloalkanoic acid to displace the halogen atom (Fig.1B).

We conducted single- and multiple-turnover enzyme reactions in $H_2{}^{18}O$ in order to examine the adequacy for these two mechanisms. The single-turnover reaction was carried out in the solution containing the enzyme in excess of substrate, whereas the multiple-turnover reaction was done by using an excess amount of substrate. If the reaction proceeds through the Fig.1B mechanism, ^{18}O is incorporated into the product both in single- and multiple-turnover

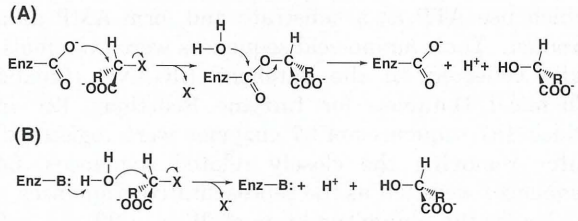

Figure 1. Proposed machanisms of L-DEX.

BIOFUNCTIONAL MOLECULES —Molecular Microbial Science—

Scope of research

Structure and function of biocatalysts, in particular, pyridoxal enzymes, NAD enzymes, and enzymes acting on xenobiotic compounds are studied to elucidate the dynamic aspects of the fine mechanism for their catalysis in the light of recent advances in gene technology, protein engineering and crystallography. In addition, the metabolism and biofunction of selenium and some other trace elements are investigated. Development and application of new biomolecular functions of microorganisms are also studied to open the door to new fields of biotechnology. For example, molecular structures and functions of thermostable enzymes and their application are under investigation.

SODA YOSHIMURA HIRASAWA
ESAKI KURIHARA

Professor: SODA, Kenji (D Agr)
Associate Professor: ESAKI, Nobuyoshi (D Agr)
Instructors: YOSHIMURA, Tohru (D Agr) KURIHARA, Tatsuo (D Eng)
Associate Instructor: HIRASAWA, Toshiko
Technicians: SEKI, Mio; NISHIKAWA, Kumiko
Guest Scholar: GORLATOV, Sergey; GORLATOVA, Natalia; NARDI-DEI, Vincenzo
Guest Research Associate: GALKIN, Andrey; LIU, Ji-Quan; JAUJOU, Muriel
Students: KUROKAWA, Yoichi (DC); JHEE, Kwang-Hwan (DC); KISHIMOTO, Kazuhisa (DC); GUTTIERREZ, Aldo Francisco (DC); CHOO, Dong-Won (DC); PARK, Chung (DC); LIU, Lidong (DC); MIHARA, Hisaaki (DC); FUCHIKAMI, Yoshihiro (DC); LI, Yongfu (DC); KITTIWORAKARN, Jongrak (DC); KURONO, Takeshi (MC); MIYAKE, Hitoki (MC); ICHIYAMA, Susumu (MC); UO, Takuma (MC); ENDO, Keiji (MC); NISHIHARA, Mitsuhiro (MC); KULAKOVA, Ludmila (MC); WATANABE, Akira (RS); SUZUKI, Takeshi; DOI, Yousuke

reactions. In the case of the Fig.1A mechanism, the single-turnover reaction causes ^{18}O-incorporation into the carboxylate group of the enzyme, but not into the product. In the multiple-turnover reaction, both the product and the carboxylate group of the catalytic residue are labeled with ^{18}O.

Under the single-turnover conditions, we found that less than 10% D-lactate produced in $H_2{}^{18}O$ contained ^{18}O, whereas under the multiple-turnover conditions, more than 95% D-lactate contained ^{18}O. These suggest that an oxygen atom of water molecule is first transferred to the enzyme, and then to the product. This supports the mechanism involving an ester intermediate shown in Fig.1A, but does not the Fig.1B mechanism, in which an oxygen atom of solvent water is directly transferred to the product.

To identify the position of the incorporated ^{18}O in the enzyme, a mutant enzyme L-DEX T15 was constructed by introducing three lysyl residues at the positions of 11, 176 and 185 of L-DEX YL by site-directed mutagenesis. Properties of the mutant L-DEX T15 such as specific activity toward L-2-chloropropionate and optimum pH were identical to those of the wild-type enzyme.

L-DEX T15 was used to carry out a multiple-turnover reaction in $H_2{}^{18}O$ with L-2-chloropropionate as a substrate. After completion of the reaction, the enzyme was digested with lysyl endopeptidase, and the resulting peptide fragments were separated on a capillary column interfaced with an ionspray mass spectrometer as a detector (PE-Sciex API *III*). When the spectrometer was in the single-quadrupole mode, the total ion current chromatogram displayed several peaks. The molecular mass of peptide 6-11 was 654.5 Da, which is approximately 4 Da higher than the predicted molecular mass (650.75 Da), although the amino acid sequence of this peptide was Gly-Ile-Ala-Phe-Asp-Lys, which is identical to that predicted from nucleotide sequence. Molecular masses of all other peptides were indistinguishable from the predicted ones. These results indicate that two ^{18}O atoms were incorporated solely into the peptide 6-11, which contains Asp10.

L-DEX T15 was incubated in $H_2{}^{18}O$ with or without L-2-chloropropionate under the multiple-turnover conditions. The enzyme was digested with lysyl endopeptidase, and the peptide 6-11 containing Asp10 was isolated with a reverse phase HPLC column. Two atoms of ^{18}O were incorporated into this peptide when L-DEX T15 was incubated in $H_2{}^{18}O$ in the presence of L-2-chloropropionate. However, ^{18}O was not incorporated when the enzyme was incubated in the absence of L-2-chloropropionate.

To identify the position of the incorporated ^{18}O more precisely, the peptide 6-11 was subjected to tandem MS/MS spectrometrical analysis. Fragmentations of the peptides were performed using mass spectrometer in the daughter ion scan mode. The parent ions of m/z 654.5 and m/z 650.2, corresponding to ^{18}O-labeled and unlabeled hexapeptides, respectively, were selected in the first quadrupole, and subjected to collision-induced fragmentation in a collision cell in the second quadrupole. The Y'' series daughter ions at m/z 484.0, 413.1, and 266.0 derived from ^{18}O-labeled peptide correspond to the fragments of Ala-Phe-Asp-Lys, Phe-Asp-Lys, and Asp-Lys, respectively. They are about 4 Da higher than those of ions at m/z 480.3, 409.1, and 262.0 of the unlabeled peptide. However, after the deletion of Asp, molecular masses of the remaining portions (Lys) of these two peptides were closely similar to each other (146.8). These results suggest that two atoms of ^{18}O of solvent water are incorporated into Asp10 of the enzyme during the dehalogenation reaction.

Accordingly, the dehalogenation reaction of L-DEX probably proceeds through the ester intermediate mechanism in which Asp10 functions as a nucleophile (Fig.1A). Since two ^{18}O atoms were incorporated into Asp10, both two oxygen atoms of the carboxylate group of Asp10 are equivalent and either can attack the substrate. The replacement of Asp10 by another amino acid residue causes significant loss in the enzyme activity (5). This also supports the involvement of Asp10 in the catalysis.

The same reaction mechanism in which a nucleophilic carboxylate group takes part has been proposed for three types of hydrolases: rat liver microsomal epoxide hydrolase (6), haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10 (7), and (4-chlorobenzoyl)coenzyme A dehalogenase from *Pseudomonas* sp. strain CBS3 (8). Epoxide hydrolase and haloalkane dehalogenase are structurally related to each other, but (4-chlorobenzoyl)coenzyme A dehalogenase does not share sequence identity with either of these two enzymes. L-DEX does not show a significant sequence similarity to any of these three enzymes. Hence, L-DEX resembles these hydrolases solely by the presence of an active site nucleophilic carboxylate.

References

1. Liu J, Kurihara T, Hasan A K M Q, Nardi-Dei V, Koshikawa H, Esaki N and Soda K, *Appl. Environ. Microbiol.* **60**, 2389-2393 (1994).
2. Nardi-Dei V, Kurihara T, Okamura T, Liu J, Koshikawa H, Ozaki H, Terashima Y, Esaki N and Soda K, *Appl. Environ. Microbiol.* **60**, 3375-3380 (1994).
3. Liu J, Kurihara T, Nardi-Dei V, Okamura T, Esaki N and Soda K, *Biodegradation* **6**, 223-227 (1995).
4. Schneider B, Muller R, Frank R and Lingens F, *Biol. Chem. Hoppe-Seyler* **374**, 489-496 (1993).
5. Kurihara T, Liu J, Nardi-Dei V, Koshikawa H, Esaki N and Soda K, *J. Biochem.* **117**, 1317-1322 (1995).
6. Lacourciere G M and Armstrong R N, *J. Am. Chem. Soc.* **115**, 10466-10467 (1993).
7. Pries F, Kingma J, Pentenga M, van Pouderoyen G, Jeronimus-Stratingh C M, Bruins A P and Janssen D B, *Biochemistry* **33**, 1242-1247 (1994).
8. Yang G, Liang P-H and Dunaway-Mariano D, *Biochemistry* **33**, 8527-8531 (1994).